IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re the application of

Michio YAMAMURA et al. Conf. No. 2444

Application No.: 10/590,986 Group Art Unit: 1623

Filed: August 29, 2006 Examiner: L. E. Crane

For: D-RIBOSE FOR IMPROVING DEPRESSION-LIKE SYMPTOMS

DECLARATION

Honorable Commissioner for
Patents and Trademarks
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

I, Toshio ASAHI, a citizen of Japan, declare and say that:

1. I have studied pharmacology in The Graduate School of Agriculture Science, Gifu University (Master Course), Veterinary Sciences, Pharmacology Course from April 1981 till March 1983 and completed the master's course at the same school in March 1983.

Since April 1983 up till July 1997, I have been an employee of Mitsubishi Tanabe Pharma Corporation (former Tanabe Seiyaku Co., Ltd.) and has been engaged in research and development of drugs for central nervous system such as antidepressants, anti-dimentia drugs as a specialist in ethopharmaology from April 1983 to July 1997. From August 1997 until the present, I have been engaged in trustee business of pharmacological experiments at Kabushiki Kaisha Tanabe R & D Services, during which I have done the trustee business with respect to ribose which has a relation with the present invention.

2. I have read the descsription and claims of the instant U.S. Patent Application No. 10/590,986 and also have read an outstanding Office Action on the merit dated October 10, 2007.

3. Under my direction, the following experiments have been done for the purpose of determining the effects of D-ribose on the brain neurotransmitter owing to the activity of D-ribose to promote ATP production since it has been well known that D-ribose has a function to promote ATP production [cf. Bio Industry, 22(6), p.64-70 (2005)].

In the following experiments, the variation of neurotransmitters such as serotonin (5-HT), dopamine (DA) and noradrenaline (NA) in the brain was measured in rats loaded with stress.

I. Materials and Methods

1) Substance

D-Ribose was provided by the Department of Fine Chemicals Department, Tanabe Seiyaku Co., Ltd.

2) Animals

Male Sprague-Dawley rats (6-week-old, weighing 200-220g, Japan SLC, Inc.) were purchased and used for the experiments after a 1-week rearing period in the animal room, the environment of which was adjusted to the following conditions: temperature of 23 ± 2°C, humidity of 30 to 70%, ventilation frequency of 12 times/h or more, and 12-h illumination (from 6:30 AM to 6:30 PM). Using the solid feed CRF-1*) (Oriental Yeast Co., Ltd.), the animals were reared with free access to drinking water until termination of the experiments. The present experiments were carried out after obtaining permission from the Committee of Animal Experimentation, Tanabe Seiyaku Co., Ltd.

*) CRF-1 was not supplemented with D-ribose during the production thereof, and in this experiment no D-ribose was added like in Examples 1 to 5 in the instant U.S. Patent Application No. 10/590,986.

3) Methods

Based on the body weight measured on the first day of the study, the animals were divided into 5 groups (n=7) as follows: 2 groups (untreated control and D-ribose-treated rats) that were kept under normal rearing conditions for 5 days, and 3 groups (stress-loaded control, stress-loaded rats treated with D-ribose, and stress-loaded rats treated with glucose) that were kept in rearing cages placed in water to a depth of about 1.5 cm at about 23°C. Both D-ribose and glucose were orally administered, at a dose of 500 mg/kg twice daily at an interval of 5

hours (each daily dose: 1,000 mg/kg) for 5 days. The dosing volume of the test substances was set to 10 mL/kg, and the same volume of distilled water was orally administered repeatedly in the untreated and stress-loaded control groups.

The animals were sacrificed by decapitation on the day after the final administration, and the brain was rapidly removed from cranium within 1 min and then immediately frozen by immersion in liquid nitrogen. With reference to the rat brain atlas by Konig and Klippel (Konig et al. 1963), the tissues of the cerebral cortex, nucleus accumbens, hypothalamus, and raphe nucleus were collected on filter papers that were immersed in saline and cooled on ice. After measuring the weight of the individual tissue samples, 2 mL of 0.4 N $HClO_4$ was added to each of the samples of cerebral cortex, nucleus accumbens, and hypothalamus, while 1 mL of 0.4 N $HClO_4$ was added to the tissue sample of raphe nucleus. The tissue samples were then homogenized by cooling on ice. After centrifugation at 2,000 rpm for 20 min, the supernatants were collected and used for monoamine determination. The supernatants were preserved at −80°C until used in the analysis.

After separating the target components, NA and DA, from the supernatants using the HPLC column switching technique, the components were treated with diphenyl ethylenediamine for fluorescence labeling. Fluorescence was then determined using a fluorometer. The target component, 5-HT, was then separated from the supernatants by the reversed-phase HPLC technique, and fluorescence was determined as before.

4) Statistical Analysis

Data were represented by mean values ± standard errors (means ± SEM). Student's t-test was used to analyze the differences between the untreated control and stress-loaded control, while Bonferroni-Dunn's multiple comparison tests were performed to analyze the inter-group difference between the stress-loaded groups. $p < 0.05$ was considered to be statistically significant.

II. Results

1) DA Concentrations in Hypothalamus and Nucleus Accumbens

DA concentrations in the hypothalamus and nucleus accumbens of rats in the respective

groups are shown in Table 1.

DA concentrations in the hypothalamus and the nucleus accumbens in the untreated control rats were 404 ± 20 μg/g tissue and 4,669 ± 417 μg/g tissue, respectively. DA concentrations in the hypothalamus and the nucleus accumbens in the stress-loaded control rats were 334 ± 19 μg/g tissue and 3,397 ± 274 μg/g tissue, respectively; significantly lower compared with those in the untreated control rats.

After administration of D-ribose to non-stress loaded rats at a daily dose of 1,000 mg/kg, no significant differences were observed in the DA concentrations of both tissues, compared with those in the untreated control rats. However, when D-ribose was administered to stress loaded rats, DA concentrations in the hypothalamus and nucleus accumbens were found to be 425 ± 15 μg/g tissue and 5,003 ± 366 μg/g tissue, respectively. This increase was significant compared with the data for the stress-loaded control rats. On the other hand, DA concentration in the nucleus accumbens in the stress-loaded rats was comparable to that in the stress-loaded control rats given glucose at a daily dose of 1,000 mg/kg; however, DA concentration in the hypothalamus showed an increasing trend compared with the data for the stress-loaded control rats.

2) 5-HT Concentration in Raphe Nucleus

The concentrations of 5-HT in the raphe nucleus of rats in the respective groups are shown in Table 1.

The concentrations of 5-HT in the raphe nucleus in the untreated control and stress-loaded control rats were 225 ± 21 μg/g tissue and 170 ± 14 μg/g tissue, respectively. The concentration of 5-HT in the raphe nucleus in the stress-loaded control rats showed a significant decrease compared with that in the untreated control rats.

In the stress-loaded rats, the concentration of 5-HT in the raphe nucleus was 237 ± 16 μg/g tissue in response to administration of D-ribose at a daily dose of 1,000 mg/kg. This was a significant increase compared with that seen in the stress-loaded control rats. The concentration of 5-HT in the raphe nucleus was not affected by either administration of D-ribose at a daily dose of 1,000 mg/kg in the rats not subjected to stress load or

administration of glucose at a daily dose of 1,000 mg/kg in the stress-loaded rats.

3) NA Concentration in Cerebral Cortex

NA concentrations in the cerebral cortex of rats in the respective groups are shown in Table 1.

NA concentrations in the cerebral cortex of the untreated control and stress-loaded control rats were 148 ± 6 μg/g tissue and 171 ± 7 μg/g tissue, respectively. NA concentration in the cerebral cortex of the stress-loaded control rats was significantly higher than that seen in the untreated control rats.

NA concentration in the cerebral cortex was found to be 143 ± 8 μg/g tissue in response to administration of D-ribose at a daily dose of 1,000 mg/kg in the stress-loaded rats; this was significantly decreased compared with the data for the stress-loaded control rats. NA concentration in the cerebral cortex was unaffected both by administration of D-ribose at a daily dose of 1,000 mg/kg in the rats not subjected to stress load and by administration of glucose at a daily dose of 1,000 mg/kg in the stress-loaded rats.

Table 1 Effect of D-Ribose on brain dopamine, noradrenaline and 5-HT concentrations in chronically stressed rats

| Agent | Stress | Dose (mg/kg/day) | Hypothalamus | Nucleus accumbens | Raphe nucleus | Frontal cortex |
|---|---|---|---|---|---|---|
| | | | dopamine ( $\mu$ g/g tissue) | dopamine ( $\mu$ g/g tissue) | serotonin (ng/g tissue) | noradrenaline ( $\mu$ g/g tissue) |
| Distilled water | − | − | 404 ± 20 | 4669 ± 417 | 225 ± 21 | 148 ± 6 |
| D-Ribose | − | 1000 | 371 ± 21 | 4507 ± 615 | 233 ± 38 | 160 ± 8 |
| Distilled water | + | − | 334 ± 19 # | 3397 ± 274 # | 170 ± 14 # | 171 ± 7 # |
| D-Ribose | + | 1000 | 425 ± 15 * | 5003 ± 366 * | 237 ± 16 * | 143 ± 8 * |
| Glucose | + | 1000 | 397 ± 31 | 3350 ± 449 | 154 ± 27 | 163 ± 9 |

Values are means ± SEM, N=7.

#: $P<0.05$ : Significantly different from Distilled water and stress (−) group (Student's t-test).

*: $P<0.05$ : Significantly different from Distilled water and stress (+) group (Bonferroni/Dunn type multiple comparison test).

As is seen from the above experimental results in the stress-loaded rats, D-ribose showed effects of increasing the amount of serotonin (5-HT) in the raphe nucleus of rats by 1.4 times larger than that in control. It has already been reported that increase of brain serotonin induces anti-depressant action [cf. Neuroscience and Biobehavioral Reviews 29 (2005) 547-569].

4. It is my opinion based upon my knowledge and experience in this field with reference to the above experimental results as well as the experimental results disclosed in Examples 1 to 5 in the instant U.S. Patent Application No. 10/590,986;

that in view of the above experimental results wherein D-ribose showed increase of brain serotonin, it will be clear that D-ribose has an anti-depressant activity owing to the increase of the brain serotonin;

that the methods used in the forced swimming test in Examples 1 and 2 in the instant U.S. Patent Application No. 10/590,986 and in the reserpine-induced hypothermia competitive test in Examples 3 and 4 in the instant U.S. Patent Application No. 10/590,986 have usually been used for developing anti-depressant drugs, and when a compound has been recognized to be effective in these experimental methods, it is considered to be useful as an anti-depressant drug, and in fact many compounds found to be effective in these methods have been used by clinical medical doctors;

that as shown in Example 5 in the instant U.S. Patent Application No. 10/590,986, it was experimentally confirmed that D-ribose exhibited anti-fatigue effects in psychological stress-loaded rats, which results will further support the anti-depressant effects of D-ribose that were confirmed by the experiments in Examples 1 to 4 in the instant U.S. Patent Application No. 10/590,986;

that in view of the experimental results as shown in Examples 1 to 5 in the instant U.S. Patent Application No. 10/590,986, it will be clear that D-ribose has an action of

improving depressive symptoms, and hence D-ribose will be able to improve depressive symptoms, such as hypobulia, general fatigue, sluggishness, enervation, deterioration in concentration, memory impairment, abnormal sensation, obtundation, impaired sight, decline in thinking power, indefinite complaint, drop in operation efficiency, feeling of malaise, and the like;

5. I know that U.S. Government has given neither "Minimum Daily Requirement" nor "Recommended Dietary Allowance" with respect to D-ribose, but I believe by my common sense that D-ribose will exhibits its activities after being intaken in the brain in view of the experimental results in Examples 1 to 4 in the instant U.S. Patent Application No. 10/590,986 as well as the effects on variation of brain neurotransmitters shown in the above experiments and in Example 5 in the instant U.S. Patent Application No. 10/590,986, regardless the mechanism thereof.

The undersigned declares further that all statements made herein of his own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the above-identified application or any patent issuing thereon.

This 20 day of February, 2008

Toshio Asahi

Toshio ASAHI